

# Surviving the Cytochrome Seas

# Minireview

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In the molecular drama of apoptotic cell death, perhaps there is no character stranger than cytochrome c. This familiar heme protein can be transformed, in Jekyll and Hyde fashion, from life-sustainer into life-destroyer. Normally, cytochrome c is restricted to the intermembrane space of mitochondria, where it plays a well-known role in the electron transport chain. In many cases, though, early in apoptosis, an ill-defined mitochondrial lesion allows this protein to escape into the cytosol (Liu et al., 1996; Bossy-Wetzel et al., 1998; Kim et al., 1997; Kluck et al., 1997; Vander Heiden et al., 1997; Yang et al., 1997; Pastorino et al., 1998). What it does there, as suggested by biochemical experiments (Liu et al., 1996; Li et al., 1997b), is to interact with the cytosolic factors Apaf-1 and procaspase-9, leading to the aggregation and transactivation of the latter. The active caspase-9, in turn, processes the “executioner” caspases (proteases important for the execution phase of apoptosis). This “killer” role for cytochrome c is further supported by the phenotype of mice nullizygous for caspase-9 (Hakem et al., 1998; Kuida et al., 1998) or Apaf-1 (Cecconi et al., 1998; Yoshida et al., 1998). These mice are defective in many (but not all) forms of apoptosis. The effects of deficiency in caspase-9 (Hakem et al., 1998; Kuida et al., 1998) or the downstream target caspase-3 are severe in the developing brain, which literally bursts through the skull due to excess neuronal mass. In animals lacking Apaf-1, there is a delay in the loss of the interdigital webs, hyperplasia of neurons and retinal cells, defects in the formation of the lens and palate, and many other developmental consequences (Cecconi et al., 1998; Yoshida et al., 1998). Unless the Apaf-1/caspase-9 pathway can somehow be activated independently of cytochrome c, these results argue for a broad (although not universal) role of cytochrome c in apoptotic cell death.

The exquisite sensitivity of cell extracts to cytochrome c has provoked a simple hypothesis that all the cell needs to do to regulate apoptosis is to control the efflux of cytochrome c from its mitochondria. In support of this idea, studies both in intact cells and in cell-free systems showed that the BCL-2 and BCL-X<sub>L</sub> proteins prevent cell death, at least in part, by preventing the translocation of cytochrome c (Kim et al., 1997; Kluck et al., 1997; Vander Heiden et al., 1997; Yang et al., 1997; Srinivasan et al., 1998). BAX, a pro-apoptotic counterpart of BCL-2 and BCL-X<sub>L</sub>, may lead to cell death through the opposite activity of promoting cytochrome c release (Juergensmeier et al., 1998; Pastorino et al., 1998).

However, the full story is not so simple, as experiments in which cytochrome c was microinjected into

cultured cells have shown (Brustugun et al., 1998; Li et al., 1997a; Srinivasan et al., 1998; Zhivotovsky et al., 1998). Cell lines were found to vary in their sensitivity to killing by microinjected cytochrome c. Even for the sensitive cells, moreover, the intracellular concentrations of cytochrome c needed for cell killing (2–10 μM) were an order of magnitude higher than those required to activate caspases in cell extracts (0.2–0.5 μM). Intact cells thus seem to have a way to regulate or suppress the response to cytosolic cytochrome c. When apoptosis is induced, the inhibitory effect is removed; this suppression is also apparently lost during the preparation of cell extracts. Are the cell-free systems merely artifactual in this regard, or is there a hint of something still to be learned here about apoptotic regulation in vivo?

This question has now been brought sharply into focus in a new study by Deshmukh and Johnson (1998 [this issue of *Neuron*]) regarding the effects of cytochrome c microinjection into primary sympathetic neurons. These cells require the continuous presence of nerve growth factor (NGF) for survival; after the withdrawal of trophic factor, they die by apoptosis within ~24 hr. Expression of the BAX protein is required for cell death following NGF deprivation, because neurons from BAX-deficient mice survive for at least several weeks in the absence of the growth factor. Cell death also requires protein synthesis, as treatment with cycloheximide (CHX) rescues the cells from death following NGF withdrawal. Since BAX expression is required for cell death following NGF withdrawal, the effect of CHX may be at the level of expression of this protein.

In this new study, sympathetic neurons maintained in NGF were found to be resistant to killing by microinjected cytochrome c. On the other hand, neurons deprived of NGF, but kept alive either because they lacked BAX or were treated with CHX, failed to release cytochrome c from their mitochondria but did die in response to cytochrome c injection. Deshmukh and Johnson conclude that there are two processes contributing to the apoptotic death of sympathetic neurons. One of these is the release of cytochrome c from mitochondria, and the other is the development of what they call “competence-to-die,” defined as the ability of the cell to undergo apoptosis in response to the presence of extramitochondrial cytochrome c. NGF signals a block to both of these processes. When NGF is withdrawn, however, the two processes occur coordinately and may be regulated independently (see Figure 1).

What does competence-to-die entail, in molecular terms? We don't really know. One could guess that competence-to-die reflects the intracellular accumulation of dATP, a cofactor of Apaf-1. However, microinjection of dATP along with cytochrome c did not confer competence-to-die on neurons cultured in NGF. Another possibility is that NGF signals the posttranslational modification of one or more of the components of the Apaf-1/caspase-9 pathway, at or downstream from the point where cytochrome c acts. However, in the presence of CHX, a 30 min treatment of trophic factor-deprived cells with NGF prior to cytochrome c injection was unable to

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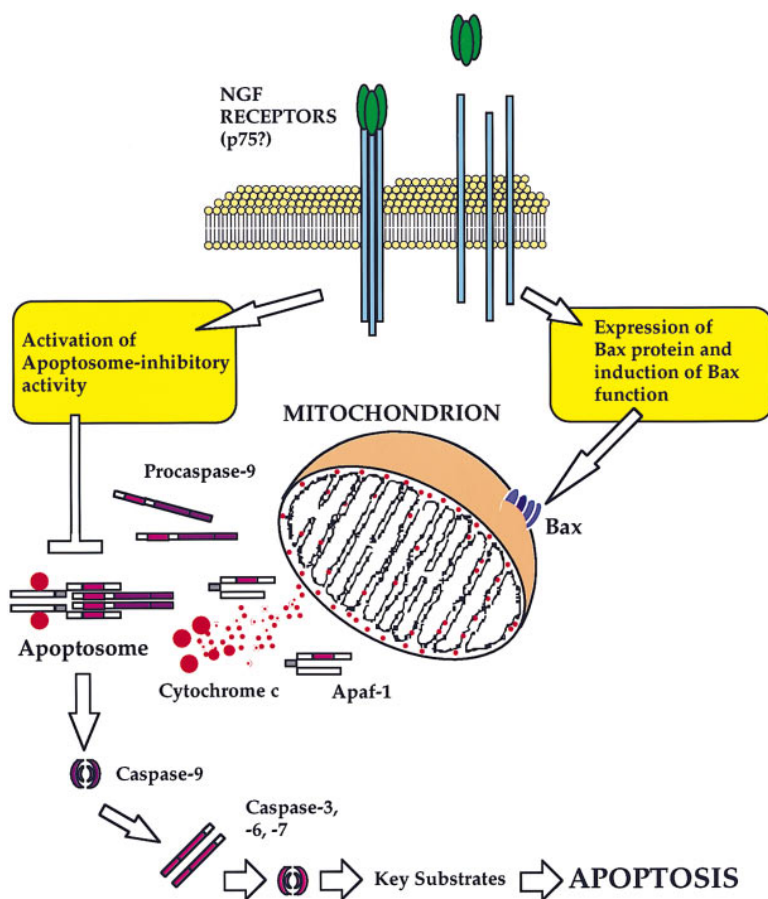


Figure 1. A Model for the Regulation of Apoptosis in Neurons

Upon withdrawal of NGF, a signal is generated (or derepressed) which depends on the expression of BAX and activates this molecule to participate in the apoptotic process. BAX translocates to mitochondria and triggers the release of cytochrome c. In the cytosol, cytochrome c associates with Apaf-1, which then binds procaspase-9 to form a vertebrate apoptosome. Procaspase-9 transactivates, and the active caspase then cleaves and activates downstream caspases (3, 6, and 7), which in turn cleave key substrates to orchestrate apoptosis. In the presence of NGF, a signal is generated that results in inhibition of apoptosome formation or function, such that cytosolic cytochrome c does not cause cell death. Presumably, this is mediated via expression of an inhibitor.

rescue the cells. Thus, the reversal of competence-to-die appears to require more than the mere activation of the NGF signaling pathway.

Logically, there are three general ways in which NGF may inhibit the susceptibility of the cell to cytosolic cytochrome c. Sustained NGF signaling might modify one of the three components (procaspase-9, Apaf-1, or cytochrome c itself), might inhibit the activity of the caspase (needed not only for downstream signaling but also for cytochrome c-triggered transactivation), or might regulate a hypothetical second signal (which is present in extracts but not in the cytosol of viable cells). Candidates for the second possibility exist in the form of the inhibitor of apoptosis proteins (IAPs), including XIAP. XIAP is a potent inhibitor of caspase-9 and can thereby interfere with its activation via cytochrome c/Apaf-1 (Deveraux et al., 1998). If the levels of these proteins are dependent on the presence of NGF, it is possible that this may account for the resistance of NGF-treated neurons to microinjected cytochrome c. It is not clear, however, why the effects of such proteins should be lost upon preparation of cell-free extracts, since they have been shown to function *in vitro*.

The death-permissive state induced by NGF withdrawal requires 16–18 hr to develop. This suggests that a slow process such as the degradation of a preexisting macromolecule is responsible. Protein synthesis is

not required, because neurons can develop competence-to-die even when CHX is present. It is likely, therefore, that upon removal of NGF, the cells cease production of the proposed inhibitor of the cytochrome c/Apaf-1/procaspase-9 apoptosome. Additionally, the NGF-deprived cells may now permit BAX activity to manifest, when it translocates to the mitochondria to trigger the release of cytochrome c. Evidence has been presented that the unligated p75 NGFR generates a signal, which is inhibited by the presence of NGF (Rabizadeh et al., 1993). If so, then it is possible that this signal is responsible for inducing BAX function.

The concept of competence-to-die gives us a possible framework for understanding the varied sensitivities of cells to microinjected cytochrome c. We can speculate that some spatially organized feature of intact cells, lost upon preparation of cytosolic extracts, such as the cytoskeleton, the endomembrane system, or the plasma membrane, contributes a signal that inhibits the proapoptotic activity of cytochrome c. Of the structured components in the cell, the endoplasmic reticulum may be the best candidate, because it has already shown to be a site for BCL-2 regulation of apoptosis (Zhu et al., 1996). This would help to explain why cells expressing BCL-2 appear to be more resistant to the effects of microinjected cytochrome c (Zivotovsky et al., 1998). In addition to inhibiting cytochrome c release, we may speculate that BCL-2 (on the ER?) inhibits cellular

changes that make a cell competent to receive the cytochrome c signal.

The simple model of apoptosis in which the release of cytochrome c from mitochondria was both necessary and sufficient for some forms of cell death was attractive and helped to explain many features of apoptosis regulation. But, like many simple models, it served its purpose and will now have to be put aside in favor of new models that accommodate emerging complexity in the system. Clearly, we have much to learn about the additional signals that regulate the response to cytosolic cytochrome c and thereby control the life and death of neurons (and probably other cells). For the present, a fog has settled over the cytochrome seas, but when it lifts who knows what marvelous new regions there will be to explore?

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